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Volatile oil from *Guarea macrophylla* ssp. *tuberculata*: Seasonal variation and electroantennographic detection by *Hypsipyla grandella*

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Abstract

GC and GC–MS analyses of the volatile oils from *Guarea macrophylla* (Meliaceae) collected during three different periods in one year (February, June and October) indicated a seasonal variation in chemical composition. Whilst sesquiterpenes were the predominant class of components present in the leaf oil, a seasonal dependent variation in the degree of oxygenation of these compounds was detected, which seemed to be associated with phenological factors. The leaf oil, and fractions thereof, were subjected to GC coupled with electroantennographic detection employing antennae of females of *Hypsipyla grandella*, an insect pest that attacks several meliaceous species. Five compounds elicited significant responses and these were identified as ledol, 1-cubenol, guai-6-en-10β-ol, 1-*epi*-cubenol, and τ-muurolol. The results suggest that these components could be responsible for the attraction of *H. grandella* to *G. macrophylla*.

Keywords: *Guarea macrophylla* tuberculata; Meliaceae; *Hypsipyla grandella*; Pyralidae; Mahogany shoot borer; Electroantennography; Volatile oils; Sesquiterpenes

1. Introduction

The Meliaceae (mahogany family) consists of 51 genera (containing nearly 1400 species) of which *Cabralea*, *Carapa*, *Cedrela*, *Trichilia*, *Guarea* and *Swietenia* are represented in Brazil. Many of the ca. 150 species of the genus *Guarea* are widely distributed (Pennington and Styles, 1975) throughout Latin America and Africa. Thus, within Brazil, *Guarea macrophylla* Vahl ssp. tuberculata occurs in the southern states of Rio Grande do Sul, Rio de Janeiro and Minas Gerais, in the central states of Mato Grosso and Brasília, and also in the Amazonian region to the north of the country. In the southern areas, the tree is confined to the lowland coastal rain forest and is often found growing alongside river banks, while in Mato Grosso and Goias it occurs mainly in gallery forests (Correa, 1984). The tree, known locally as "Ataúba", blossoms in the summer months between October and February, but mainly in November and December, and bears fruit in the winter from June to October.

Several phytochemical studies on *G. macrophylla* have been published, and a wide variety of secondary metabolites, including sequi-, di- and tri-terpenes, have been identified (Lago et al., 2000; Lago and Roque, 2002b). With respect to volatile compounds, one monoterpene,

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sixteen sesquiterpenes and six diterpenes were reported in the leaf oil (Lago and Roque, 2002a), whilst 24 sesquiterpenes were identified in the oil from the fruit (Lago et al., 2005). However, no data concerning the dynamics of the composition of the volatile oils have been presented.

The mahogany shoot borer, *Hypsipyla grandella* (Zeller, 1848) (Lepidoptera: Pyralidae), is one of the most economically important pests of Neotropical forests since it attacks the terminal shoots of plants of the family Meliaceae (Schabel et al., 1999). However, the volatile compounds of *G. macrophylla* have not been the subject of electroantennographic studies involving *H. grandella*, even though this tree is attacked by the insect larvae. Thus, with the aim of investigating the nature of the interaction between the shoot borer and *G. macrophylla*, we have studied the variation in the composition of the volatile oil of the leaves in three different periods (February, June and October) of one year, and established the selectivity and sensitivity of the antennae receptors of the female moths to the leaf volatiles.

2. Results and discussion

The total yield of volatile oil obtained from each batch of *G. macrophylla* leaves harvested in different months of the year was approximately 0.05%. Table 1 presents the mean relative percentage of each of the components of the oil of leaves collected from the same tree at four different times during the days of 15th February, 15th June and 15th October 2000. These data indicate that the leaf oils were mainly composed of sesquiterpenes, but that the relative amounts of sesquiterpene hydrocarbons and oxygenated sesquiterpenes varied considerably. Thus, during February (the sterile period) the relative level of oxygenated sesquiterpenes was 38%, but this fell to 29.2% during fruiting (October). Over the same period, the relative level of sesquiterpene hydrocarbons was 24%, but this rose with fruit development to ca. 36% and 38.8%, respectively, in June and October, suggesting that some phenological effect could be involved in this variation (Lopes et al., 1997). In February, the oxygenated sesquiterpene guai-6-en-10β-ol (**18**) was the major component of the oil (16 ± 1%) (see

Table 1
Variation in the composition of the volatile oil of leaves of *Guarea macrophylla* during one year

	Compounds	Kovats retention index	Relative percentage composition[a]		
			February	June	October
1	α-Terpineol	1189	0.13 ± 0.05	0.22 ± 0.03	–
2	α-Cubebene	1351	0.25 ± 0.02	0.26 ± 0.02	0.12 ± 0.07
3	α-Ylangene	1372	0.9 ± 0.1	1.4 ± 0.2	1.56 ± 0.05
4	α-Copaene	1376	0.54 ± 0.04	0.9 ± 0.4	0.51 ± 0.05
5	α-Gurjunene	1409	0.40 ± 0.07	0.50 ± 0.03	0.16 ± 0.09
6	β-Caryophyllene	1418	2.0 ± 0.2	2.7 ± 0.4	3.6 ± 0.2
7	α-Humulene	1454	0.9 ± 0.2	1.5 ± 0.3	1.82 ± 0.07
8	*allo*-Aromadendrene	1461	2.3 ± 0.3	3.4 ± 0.2	2.5 ± 0.1
9	Germacrene-D	1480	1.6 ± 0.2	3.1 ± 0.2	3.5 ± 0.1
10	Bicyclogermacrene	1496	6.5 ± 0.6	7.3 ± 0.3	7.0 ± 0.6
11	γ-Cadinene	1513	8 ± 1	12.8 ± 0.9	16.1 ± 0.6
12	α-Cadinene	1524	1.7 ± 0.2	1.84 ± 0.08	2.0 ± 0.2
13	Palustrol	1561	1.8 ± 0.1	1.71 ± 0.10	1.5 ± 0.2
14	Germacradien-4-ol	1574	1.0 ± 0.3	1.2 ± 0.1	1.06 ± 0.07
15	Spathulenol	1576	1.8 ± 0.7	1.0 ± 0.1	0.77 ± 0.06
16	Ledol	1629	8.9 ± 0.4	8.7 ± 0.6	6.6 ± 0.2
17	1-Cubenol	1611	3.3 ± 0.6	3.1 ± 0.2	4.7 ± 0.1
18	Guai-6-en-10β-ol	1654	16 ± 1	14 ± 1	10.4 ± 0.6
19	1-*epi*-Cubenol	1629	1.8 ± 0.1	1.4 ± 0.1	1.9 ± 0.1
20	τ-Cadinol	1637	1.6 ± 0.2	1.5 ± 0.1	1.2 ± 0.1
21	τ-Muurolol	1651	1.64 ± 0.07	1.4 ± 0.1	1.1 ± 0.1
22	Isopimara-7,15-diene	1926	–	0.07 ± 0.04	–
23	Manoyl oxide	1989	4.0 ± 0.6	3.9 ± 0.4	5.9 ± 0.4
24	Isopimara-7,15-dien-3β-ol	2161	0.07 ± 0.07	0.03 ± 0.03	–
25	Isopimara-7,15-dien-3-one	2279	4.5 ± 0.3	2.6 ± 0.3	3.8 ± 0.3
26	Isopimara-7,15-dien-2α-ol	2284	0.4 ± 0.2	0.13 ± 0.08	–
27	Labda-8,13-(*E*)-dien-15-ol	2412	0.13 ± 0.08	0.07 ± 0.04	–
Total monoterpenes			0.13 ± 0.05	0.22 ± 0.03	–
Total sesquiterpene hydrocarbons			24 ± 1	36 ± 2	38.8 ± 0.8
Total oxygenated sesquiterpenes			38 ± 1	34 ± 2	29.2 ± 0.7
Total diterpene hydrocarbon			–	0.07 ± 0.04	–
Total oxygenated diterpenes			10 ± 1	6.8 ± 0.8	9.7 ± 0.6

[a] Mean values (±standard deviation) for leaf oils collected at four different times on the same day and analysed separately ($n = 3$).

Table 1, for compound numbers). In June, although the accumulation of sesquiterpene hydrocarbons in the oil had increased, **18** remained the major constituent ($14 \pm 1\%$). In October, sesquiterpene hydrocarbons were the predominant volatile compounds, with γ-cadinene (**11**) becoming the main component present ($16.1 \pm 0.6\%$). During this period, the oil obtained from the fruit contained high concentrations of sesquiterpene hydrocarbons, with viridiflorene, γ-cadinene and cadina-1,4-diene being the main components (Lago et al., 2005).

The effects of different concentrations of leaf oil (and fractions derived therefrom) of *G. macrophylla* on antennae from female and male specimens of *H. grandella* are shown in Figs. 1 and 2, respectively. At all concentrations tested the electroantennographic responses were significantly higher for the volatile oil samples than for the control (hexane). At concentrations of 10 and 100 mg/ml, females and males showed levels of antennal activity towards the sesquiterpene hydrocarbon fraction that were significantly lower than the responses obtained from the crude oil or the oxygenated sesquiterpene fraction (there being no statistical difference between the responses to the latter two samples). Similar differences have also been noted in studies carried out with other, unrelated, insects including *Yponomeuta* sp. (Lepidoptera: Yponomeutidae), *Anthonomus grandis* (Coleoptera: Curculionidae) and *Megastigmus spermotrophus* (Hymenoptera: Torymidae) (Van Der Pers, 1981; Dickens et al., 1984; Thiéry and Marion-Poll, 1998). These results indicate that adults of *H. grandella* recognize some compounds present in the volatile oil of *G. macrophylla*; that is, the antennae of both sexes of this insect must possess receptor neurons (chemoreceptors) in sensillas that detect components of the leaf oil.

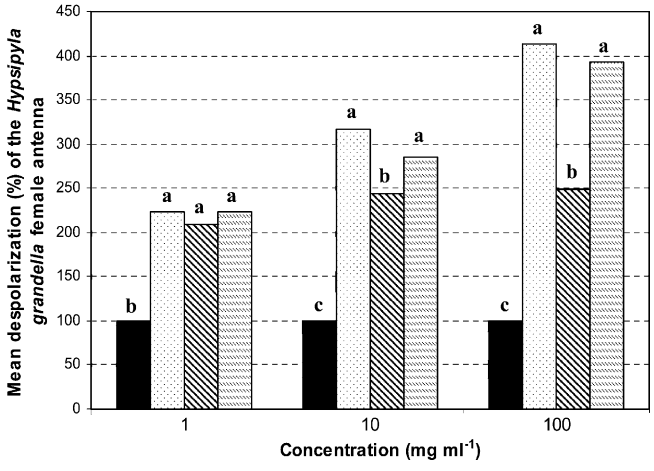

Fig. 1. Electroantennographic responses [expressed as mean percentage values with respect to the control (■ hexane = 100%)] elicited from antennae of *H. grandella* females by the crude volatile oil □, a sesquiterpene hydrocarbon fraction ▨ and an oxygenated sesquiterpene fraction ▤ from *G. macrophylla* at concentrations of 1, 10 and 100 mg/ml. [Mean values labelled with the same letter are not significantly different at $P < 0.05$ on the basis of the Tukey test ($n = 10$ antennae)].

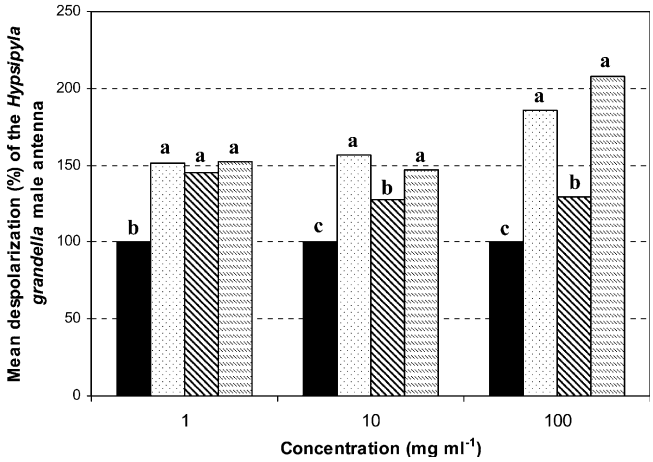

Fig. 2. Electroantennographic responses [expressed as mean percentage values with respect to the control (■ hexane = 100%)] elicited from antennae of *H. grandella* males by the crude volatile oil □, a sesquiterpene hydrocarbon fraction ▨ and an oxygenated sesquiterpene fraction ▤ from *G. macrophylla* at concentrations of 1, 10 and 100 mg/ml. [Mean values labelled with the same letter are not significantly different at $P < 0.05$ on the basis of the Tukey test ($n = 10$ antennae)].

Figs. 1 and 2 also demonstrate that the responses to the volatile extracts obtained with antennae from females were almost twice as large as those attained with male antennae. Furthermore, the dose–response exhibited by female antennae was more pronounced than that observed with antennae from males. It is thus suggested that, whilst the volatile oils from *G. macrophylla* are general attractants, they may also play a specific role in host plant and oviposition selection in agreement with the study of Maia et al. (2000). From EAG experiments, these authors claimed that the high selectivity of antennae of *H. grandella* females to the essential oils from leaves of *Cedrela odorata* and *Toona ciliata* indicated their potential roles as chemical messengers for habitat location and oviposition.

The oxygenated sesquiterpene fraction of the leaf oil of *G. macrophylla* was subjected to GC analysis with electroantennographic detection (GC–EAD) using an antenna from a *H. grandella* female. The antennal olfactory system clearly showed differential sensitivity to several compounds present in the volatile oil as depicted in Fig. 3. The main constituents producing significant antenna responses were identified as ledol (**16**), 1-cubenol (**17**), guai-6-en-10β-ol (**18**), 1- *epi*-cubenol (**19**), and τ-muurolol (**21**). Since the relative amounts of oxygenated sesquiterpenes increases during the sterile period, it is suggested that these components may play a role in attracting *H. grandella* females to *G. macrophylla* leaves at this time for feeding and/or ovipositing.

A number of studies have demonstrated the role of plant volatiles in the orientation of species of moths to their host plants. Host volatiles, particularly terpenoids, have potential importance in the feeding and/or mating behaviour of polyphagous insects, and exhibit various biological activities such as herbivore attractants, repellents, and feeding

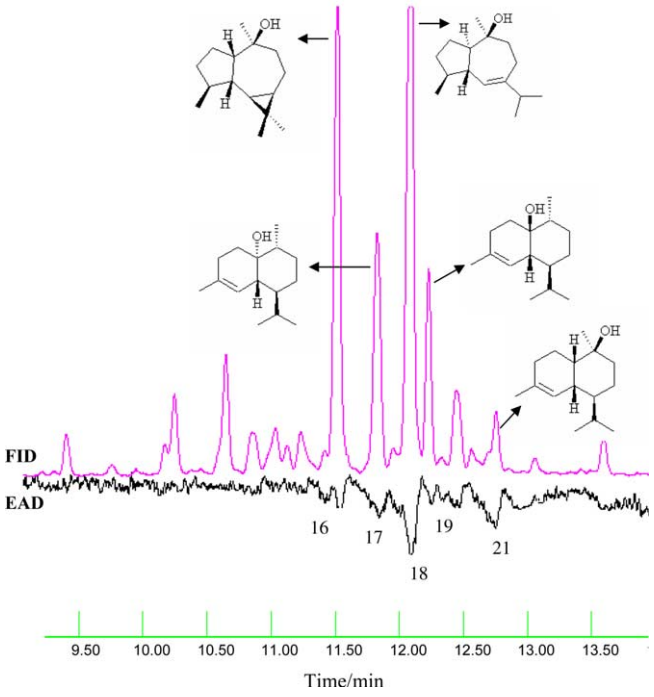

Fig. 3. GC–FID profile (upper trace) of the oxygenated sesquiterpene fraction (1.0 mg/ml) of the leaf oil of *G. macrophylla*, and the simultaneous GC–EAD response (lower trace) of antennae of *H. grandella* females. The numbered peaks elicited electrophysiological responses and were identified as **16**, ledol; **17**, 1-cubenol; **18**, guai-6-en-10β-ol; **19**, 1-*epi*-cubenol; and **21**, τ-muurolol.

stimulants (Gershenzon and Croteau, 1991; Harborne, 1991; Langenheim, 1994). Studies concerning the interaction between *H. grandella* and the mahogany tree, *Swietenia macrophylla* (Meliaceae), have shown that the sesquiterpene hydrocarbon β-caryophyllene is the main constituent responsible for the antennal response (Soares et al., 2003) and is associated with the attraction of *H. grandella* to oviposit on the leaves of this plant.

The effects of environmental and phenologic factors should be related to the accumulation of different components in the plant. Such mechanisms are specific to each species and probably associated with the attack of the insect on the plant. In the case of *G. macrophylla*, since the amounts of oxygenated sesquiterpenes in the leaves decrease during the fruiting period, there may be a reduced interaction between the volatile oil compounds and *H. grandella* at this time. In summary, the present findings provide additional information concerning the chemistry of the *Guarea–Hypsipyla* relationship, and signify that the behavioural role of these allelochemicals require further detailed investigation.

3. Experimental

3.1. Plant material and extraction of volatile oil

Fresh leaves of a single specimen of *G. macrophylla*, growing in the Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil, were collected at 08.00, 12.00, 16.00 and 20.00 h on 15th February 2000 from the tree without fruit, and at the same times on 15th June and 15th October 2000 when the tree was bearing fruit. A voucher specimen of the plant has been deposited in the Instituto de Biociências, Universidade de São Paulo. Volatile oil was obtained from fresh leaves (400 g) by hydrodistillation for 4 h using a Clevenger-type apparatus (yield ca. 200 mg).

3.2. Quantitative analysis

Volatile oils were analysed using a Hewlett–Packard (HP) 5890 series II gas chromatograph equipped with an FID detector, an automatic injector (HP 7673) and an electronic integrator (HP3396A). An HP-5 capillary column (30 m × 0.32 mm, I.D.; 0.25 μm film thickness) of cross-linked 5% phenyl–methyl silicone was employed with helium as the carrier gas at a flow rate of 1 ml/min. Temperature programming was performed as follows: 100 °C isothermal for 2 min, then increased from 100 to 240 °C at 5 °C/min, and finally isothermal at 240 °C for 5 min. The injector and detector temperatures were 180 and 260 °C, respectively. Samples were analysed in triplicate, using *n*-nonane (Sigma) as internal standard, and component concentrations were calculated from the relative GC peak areas as shown in Table 1.

3.3. Qualitative analysis

GC–MS analyses were carried out using an HP model 5973 MS coupled to an HP-5890 gas chromatograph fitted with an HP-5 column (30 m × 0.25 mm, I.D., 0.25 μm film thickness). The chromatographic conditions outlined above were employed, and the EI/MS spectra were recorded at 70 eV. Components were identified on the basis of their retention times in comparison to those determined when the oil was analysed previously (Lago and Roque, 2002a) and by co-injection with appropriate authentic samples. Additionally, the Kovats retention index for each component was determined relative to the retention times of a series of *n*-alkanes (Table 1).

3.4. Fractionation of the volatile oil

A portion (50 mg) of the crude volatile oil (collected at 12.00 h on 15th October 2000) was separated into two fractions by SiO_2 prep. TLC using CH_2Cl_2 as eluent. The fractions were extracted with CH_2Cl_2 and shown (by GC and GC–MS) to contain, respectively, sesquiterpene hydrocarbons (33 mg) and oxygenated sesquiterpenes (7 mg).

3.5. Insects

Specimens of *H. grandella* were obtained from the Entomology Laboratory of the Faculdade de Ciências Agrárias do Pará, Belém, PA, Brazil, and maintained in the labora-

tory on mahogany foliage. Pupae were established and sexed (Parra, 1986) in the Insect Bioassay Laboratory, Universidade Federal de São Carlos, São Carlos, SP, Brazil. Male and female pupae were placed separately into plastic vials (6×6 cm I.D.) and incubated in a chamber at 25 ± 1 °C and $60 \pm 5\%$ relative humidity under a 12 light:12 dark illumination regime.

3.6. Electroantennographic analyses

Electroantennographic experiments (EAG) and analyses employing electroantennographic detection (EAD) were carried out using male and female moths 1–2 days after emergence. The complete antenna was excised using biological forceps, and a few segments removed from both the base and the tip (Bjostad, 1998). The antenna was then fixed between two stainless steel electrodes by inserting the base and tip into droplets of Spectra 360® electrode gel (Parker, Orange, NJ, USA) that had been applied to each of the metal surfaces. Antennal responses were amplified and recorded using a Syntech (Davis, CA, USA) intelligent data acquisition controller interfaced to an AT486PC running EAG software.

In order to evaluate the EAG response, a filter paper (about 0.8 cm^2) impregnated with 10 µl of a freshly prepared hexane solution of the test sample, or with 10 µl of hexane as negative control, was placed into a Pasteur pipette, the tip of which was be positioned within a continuous stream of humidified and purified air that passed over the mounted antenna at a flow rate of 1.2 l/min. EAGs were obtained by releasing the test sample or hexane control, in the form of a 0.3 s flush of the pipette, into the air stream. Control stimulations were made at the beginning and at the end of each series of EAG experiments, and the test samples were applied randomly to the antenna at intervals of 60 s. Owing to the large differences in overall sensitivities between individual antennae, and to compensate for the decline in the sensitivity of an antenna during a measuring session, EAG amplitudes recorded in response to the test samples were normalised with respect to the control responses. In this normalization process, performed automatically by the Syntech EAG software, responses to the initial and final controls were defined as 100%, and values obtained between these two reference points were calculated by linear interpolation. The essential oil or oil fractions were tested on ten antennae each from *H. grandella* females. The mean normalized responses of the different compounds were submitted to ANOVA for statistical analysis and compared by the Tukey test ($P < 0.05$).

3.7. GC–FID coupled with EAD

GC analyses with electroantennographic detection (GC–EAD) were performed on a Shimadzu GC-17A instrument equipped with a Supelcowax 10 column (30 m × 0.25 mm I.D.; 0.25 µm film thickness). The chromatographic parameters outlined above were employed. The column effluent was split equally between FID and EAD detectors: for the latter, a mounted antenna was positioned such that a stream of humidified air could direct the output from the GC over the antenna/electrode assembly.

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